Disruption of *mpl* Activates β-Lactamase Production in *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa* Clinical Isolates.

Karina Calvopiña[1], Matthew B. Avison[1]*

[1]School of Cellular & Molecular Medicine, University of Bristol, Bristol, United Kingdom.

***Correspondence to: School of Cellular & Molecular Medicine, University of Bristol, Bristol, United Kingdom. matthewb.avison@bris.ac.uk**

RUNNING TITLE

Mpl loss and β-lactamase production

ABSTRACT

The hyperproduction of chromosomally encoded β-lactamases is a key method of acquired resistance to ceftazidime, aztreonam, and when seen in backgrounds having reduced envelope permeability, carbapenems. Here we show that loss of Mpl, a UDP-muramic acid/peptide ligase, is a common and previously overlooked cause of chromosomally encoded β-lactamase hyperproduction in clinical isolates of *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa*, important pathogens notorious for their β-lactam resistant phenotypes.

TEXT

Stenotrophomonas maltophilia clinical isolates are resistant to almost all β-lactams because of the production of two β-lactamases: L1, a subclass B3 metallo-β-lactamase and L2, a class A extended spectrum β-lactamase (1) Production of L1 and L2 is co-ordinately controlled by AmpR, a LysR-type transcriptional activator and induced during β-lactam challenge of cells (2). Where previously characterised, AmpR regulators have been shown to bind two ligands in a competitive manner (3, 4). As summarised in **Figure 1**, the AmpR activator ligand, an anhydro-muramyl-penta-peptide is produced during β-lactam challenge via the concerted actions of lytic transglycosylases, which release N-acetylglucosamine-anhydro-muramyl-peptides from peptidoglycan (5) and AmpG, a permease that transports them into the cytoplasm (6, 7). NagZ, an enzyme that removes the N-acetylglucosamine moiety is also necessary to release the AmpR activator ligand in some species (8), though not in *S. maltophilia* (9). The AmpR repressor ligand is a UDP-muramyl-penta-peptide (10). It is produced

via sequential addition of amino acids to a UDP-muramyl substrate, via four separate ligase enzymes, MurC (11), MurD (12), MurE (13) and MurF (14), with the last adding a D-alanine/D-alanine dipeptide made by a fifth ligase enzyme, Ddl (15). Mpl is an enzyme that can ligate a ready-made penta-peptide onto the UDP-muramyl substrate, skipping the MurC, D, E, Ddl and MurF ligation reactions, each of which requires ATP hydrolysis (16). This Mpl catalysed reaction therefore saves considerable amounts of energy for the cell. Its penta-peptide substrate comes from breakdown of anhydro-muramyl-penta-peptides by the peptide amidase AmpD. In this way, breakdown of the anhydro-muramyl-penta-peptide AmpR activator ligand by AmpD is also directly linked to production of the UDP-muramyl-penta-peptide AmpR repressor ligand by Mpl (2, 5, 17, 18) (**Fig. 1**).

Ceftazidime is a relatively weak substrate for both L1 and L2 β-lactamases from *S. maltophilia*, and so many clinical isolates remain ceftazidime susceptible (1). However, mutants that have acquired ceftazidime resistance can easily be identified in the laboratory, and ceftazidime resistant isolates are commonly encountered in the clinic. In most cases, these mutants hyperproduce L1 and L2 (19). Mutations that reduce AmpD function are known to boost L1/L2 production, because the AmpR activator ligand is broken down much less if AmpD is damaged (20). Mutations that (presumably) increase peptidoglycan turnover, releasing more muropeptides, also activate L1/L2 production, e.g. those in PBP1A, encoded by *mcrA* (21) and in the lytic transglycosylase MltD, because this mutation stimulates the net production of lytic transglycosylase activity in the cell (22). Mutations in AmpR also activate L1/L2 production (4). We have previously characterised ceftazidime resistant, β-lactamase hyper-producing laboratory selected mutants derived from the extremely well studied clinical isolate K279a. One of these mutants, KCAZ14, was wild-type for *ampR*, *ampD*, and *mcrA* (19). To identify the mutation responsible, whole genome resequencing was performed by MicrobesNG (Birmingham, UK) on a HiSeq 2500 instrument (Illumina, San Diego, CA, USA). Reads were trimmed using Trimmomatic (23) and assembled into contigs using SPAdes 3.10.1 (http://cab.spbu.ru/software/spades/). Assembled contigs were mapped to reference genome for *S. maltophilia* K279a (24) obtained from GenBank (accession number NC_010943) using progressiveMauve alignment software (25). The only mutation identified in KCAZ14 was a deletion of 18 nucleotides in *mpl* gene, deleting amino acids 141-146 of Mpl. The level of β-lactamase production, measured as previously (19) was similar for the *mpl* mutant KCAZ14, for an *ampD* loss of function mutant KCAZ10 (19) and for KM11, an *ampR* activatory mutant (4) (**Table 1**). To confirm involvement of *mpl* loss in the β-lactamase hyper-producing, ceftazidime resistant phenotype of KCAZ14, we attempted complementation *in trans*. K279a *mpl* was amplified by PCR as previously (19) with primers mpl_F (5'-ACCAGATCCAGGTACCGCC-3'), mpl_R (5'-TCTCACATCCCGTGTAGGACT-3'). The product was blunt-end ligated into pBBRMCS-5 (Gm^R) (26, 27) digested with SmaI and the resulting recombinant plasmid used to transform KCAZ14 to gentamicin resistance (15 $\mu g.mL^{-1}$) via electroporation. The ceftazidime MIC against KCAZ14(pBBRMCS-5) was 64 $\mu g.mL^{-1}$ and reduced to

4 μg.mL^{-1} in KCAZ14(pBBRMCS-5::*mpl*), the same as the MIC against wild-type K279a. Production of β-lactamase was also reduced to wild-type levels in KCAZ14(pBBRMCS-5::*mpl*) (**Table 1**) adding further confirmation of successful complementation.

We have four ceftazidime resistant, β-lactamase hyperproducing clinical *S. maltophilia* clinical isolates in our collection: isolates 49-6147, 3800 and 98 (19) and ULA-511 (28) **(Table1)**. Isolate 98 has an Insertion Sequence element disrupting *ampD* (19). Whilst we also found a mutation causing an Ala85Gly change in Mpl, the same mutation is carried by ~5% of *S. maltophilia* genomes in the Genbank database so is probably insignificant. The other three clinical isolates have *mpl* mutations. In 49-6147, the mutation causes the deletion of amino acids 92-109, which disrupts the conserved Ser-Gly-Pro region (29). In 3800, there is a frameshift at codon 368 and in ULA-511 there is a nonsense mutation at codon 360.

The result of Mpl loss in KCAZ14 and these clinical isolates will be a build-up of penta-peptides released by AmpD (**Fig. 1**). Even though there are other enzymes that can break down these penta-peptides, it seems reasonable to hypothesise that this net accumulation of penta-peptide will affect AmpD activity by feedback inhibition, increasing the concentration of its substrate, the AmpR activator ligand, causing β-lactamase hyper-production (18).

This is the first report of *mpl* disruption causing β-lactamase hyperproduction in *S. maltophilia,* and to find it in 3/4 clinical isolates was striking. It is also interesting to find that *mpl* loss of function mutations have been seen to accumulate in *Pseudomonas aeruginosa* populations carried by people with Cystic Fibrosis during long term colonisation in two separate studies (30, 31) and also in 3/4 patients with *P. aeruginosa* mediated ventilator associated pneumonia (32). Indeed, *mpl* mutation has been identified as a cause of AmpC β-lactamase hyperproduction in one *P. aeruginosa* PAO1 laboratory selected transposon-insertion mutant (33). Whilst this did not dramatically increase β-lactam MICs (33), PAO1 is relatively permeable to β-lactams, because it lacks many of the efflux pump/porin altering mutations seen in clinical isolates (34). Therefore, it would seem reasonable to propose that these clinically acquired *P. aeruginosa mpl* mutations are being selected by β-lactam therapy. We have a small collection of ceftazidime resistant *P. aeruginosa* clinical isolates, of which 2/5 have previously been confirmed to hyperproduce AmpC (35). Both have a mutation in *mpl*, according to whole genome sequencing. The mutations in isolates 86-14571 and 73-56826 cause Met297Val and an Arg103His changes in Mpl, respectively. We conclude, therefore, that *mpl* loss in *S. maltophilia* and *P. aeruginosa* is a clinically important and previously under-reported cause of β-lactamase hyperproduction and acquired β-lactam resistance.

ACKNOWLEDGEMENTS

This work was funded, in part, by grant MR/N013646/1 to M.B.A from the Antimicrobial Resistance Cross Council Initiative supported by the seven United Kingdom research councils. K.C. was in receipt of a postgraduate scholarship from SENESCYT, Ecuador.

CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

Fig 1

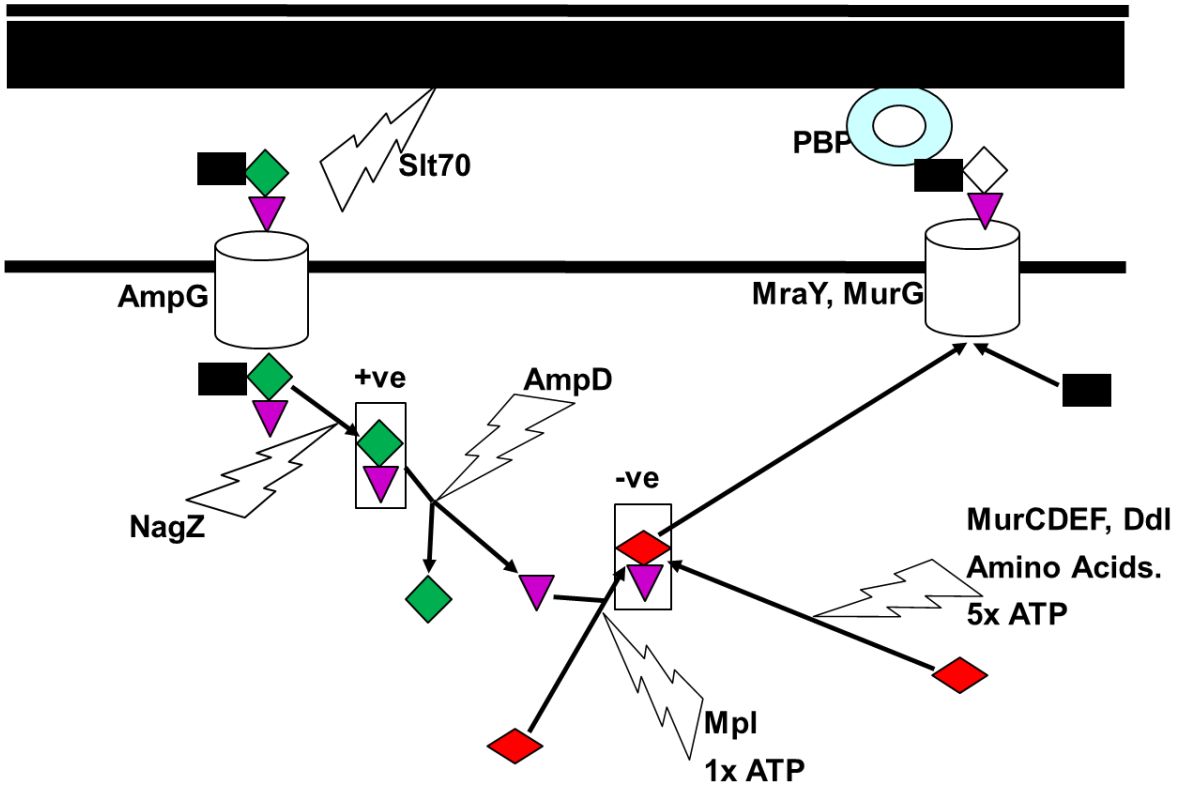

Fig 1 Role of Mpl in peptidoglycan recycling and AmpR activation.

The schematic shows that N-acetylglucosamine (black square)-anhydro-muramyl (green diamond)-penta-peptide (purple triangle) is removed from peptidoglycan by lytic transglycosylases such as Slt70 and enters the cytoplasm through the permease AmpG. NagZ removes the N-acetylglucosamine group to produce the anhydro-muramyl-penta-peptide AmpR activator ligand ("+ve"). AmpD then releases the penta-peptide ready to be linked to a UDP-muramic acid molecule (red diamond) by Mpl to produce the UDP-muramyl-penta-peptide AmpR repressor ligand ("-ve"). This can then be further incorporated into the biosynthetic pathway and processed by MurG and MraY, which add N-acetylglucosamine and penicillin binding proteins, which add these high energy N-acetylglucosamine-muramyl (white diamond)-penta-peptide substrates to the nascent peptidoglycan strand. UDP-muramyl-penta-peptide formation can also occur without peptidoglycan recycling, through the sequential addition of amino acids to UDP-Muramic acid. However, this requires five moles of ATP per mole of UDP-muramyl-penta-peptide, whilst the recycling pathway only requires one.

Table 1

β-Lactamase activity (nmol.min^{-1}.μg^{-1} protein nitrocefin hydrolysed in cell extracts) observed in *S. maltophilia* K279a and in ceftazidime resistant K279a mutants and clinical isolates carrying different mutations.

Isolate	Mean β-lactamase activity ±SEM	Relevant amino acid changes (Relative to K279a)
K279a	0.02±0.004	WT
KM11	0.99±0.03	Asp135Asn in AmpR
KCAZ10	1.52±0.04	159-168del in AmpD
KCAZ14	0.72±0.01	140-146del in Mpl
49-6147	0.45±0.12	92_109del Mpl
3800	0.73±0.03	Truncation at 368 in Mpl
98	1.76±0.07	IS insertion in *ampD*; Ala85Gly* in Mpl
ULA-511	1.19±0.01	Truncation at 360 in Mpl
KCAZ14 (pBBRMCS-5)	1.14±0.10	
KCAZ14 (pBBRMCS-5::*mpl*)	0.03±0.003	

*Random Genetic Drift

WT: Wild type

REFERENCES

1. **Calvopina K, Hinchliffe P, Brem J, Heesom KJ, Johnson S, Cain R, Lohans CT, Fishwick CWG, Schofield CJ, Spencer J, Avison MB.** 2017. Structural/mechanistic insights into the efficacy of nonclassical beta-lactamase inhibitors against extensively drug resistant *Stenotrophomonas maltophilia* clinical isolates. Molecular Microbiology **106:**492-504.
2. **Jacobs C, Frere JM, Normark S.** 1997. Cytosolic intermediates for cell wall biosynthesis and degradation control inducible beta-lactam resistance in Gram-negative bacteria. Cell **88:**823-832.
3. **Kraft AR, Prabhu J, Ursinus A, Holtje JV.** 1999. Interference with murein turnover has no effect on growth but reduces beta-lactamase induction in *Escherichia coli*. Journal of Bacteriology **181:**7192-7198.
4. **Okazaki A, Avison MB.** 2008. Induction of L1 and L2 beta-lactamase production in *Stenotrophomonas maltophilia* is dependent on an AmpR-type regulator. Antimicrobial Agents and Chemotherapy **52:**1525-1528.
5. **Vadlamani G, Thomas MD, Patel TR, Donald LJ, Reeve TM, Stetefeld J, Standing KG, Vocadlo DJ, Mark BL.** 2015. The beta-lactamase gene regulator AmpR is a tetramer that recognizes and binds the D-Ala-D-Ala motif of its repressor UDP-N-acetylmuramic acid (MurNAc)-pentapeptide. Journal of Biological Chemistry **290:**2630-2643.
6. **Lindquist S, Westonhafer K, Schmidt H, Pul C, Korfmann G, Erickson J, Sanders C, Martin HH, Normark S.** 1993. AmpG, a signal transducer in chromosomal beta-lactamase induction. Molecular Microbiology **9:**703-715.
7. **Huang YW, Lin CW, Hu RM, Lin YT, Chung TC, Yang TC.** 2010. AmpN-AmpG Operon is essential for expression of L1 and L2 beta-lactamases in *Stenotrophomonas maltophilia*. Antimicrobial Agents and Chemotherapy **54:**2583-2589.
8. **Votsch W, Templin MF.** 2000. Characterization of a beta-N-acetylglucosaminidase of *Escherichia coli* and elucidation of its role in muropeptide recycling and beta-lactamase induction. Journal of Biological Chemistry **275:**39032-39038.
9. **Huang YW, Hu RM, Lin CW, Chung TC, Yang TC.** 2012. NagZ-dependent and NagZ-independent mechanisms for beta-lactamase expression in *Stenotrophomonas maltophilia*. Antimicrobial Agents and Chemotherapy **56:**1936-1941.
10. **Uehara T, Park JT.** 2002. Role of the murein precursor UDP-N-acetylmuramyl-L-Ala-gamma-D-Glu-meso-diaminopimelic acid-D-Ala-D-Ala in repression of beta-lactamase induction in cell division mutants. Journal of Bacteriology **184:**4233-4239.
11. **Falk PJ, Ervin KM, Volk KS, Ho HT.** 1996. Biochemical evidence for the formation of a covalent acyl-phosphate linkage between UDP-N-acetylmuramate and ATP in the *Escherichia coli* UDP-N-acetylmuramate:L-alanine lipase-catalyzed reaction. Biochemistry **35:**1417-1422.
12. **Pratvielsosa F, Menginlecreulx D, Vanheijenoort J.** 1991. Over-production, purification and properties of the uridine-diphosphate N-acetylmuramoyl-L-alanine-D-glutamate ligase from *Escherichia coli*. European Journal of Biochemistry **202:**1169-1176.
13. **Michaud C, Menginlecreulx D, Vanheijenoort J, Blanot D.** 1990. Over-production, purification and properties of the uridine-diphosphate-N-acetylmuramoyl-L-alanyl-D-glutamate-meso-2,6-diaminopimelate ligase from *Escherichia coli*. European Journal of Biochemistry **194:**853-861.
14. **Duncan K, Vanheijenoort J, Walsh CT.** 1990. Purification and characterization of the D-alanyl-D-alanine adding enzyme from *Escherichia coli*. Biochemistry **29:**2379-2386.
15. **Zawadzke LE, Bugg TDH, Walsh CT.** 1991. Existence of two D-alanine-D-alanine ligases in *Escherichia coli* - cloning and sequencing of the *ddlA* gene and purification and characterization of the DdlA and DdlA enzymes. Biochemistry **30:**1673-1682.
16. **MenginLecreulx D, vanHeijenoort J, Park JT.** 1996. Identification of the *mpl* gene encoding UDP-N-acetylmuramate:L-alanyl-gamma-D-glutamyl-meso-diaminopimelate ligase in *Escherichia coli* and its role in recycling of cell wall peptidoglycan. Journal of Bacteriology **178:**5347-5352.
17. **Park JT, Uehara T.** 2008. How bacteria consume their own exoskeletons. Microbiology and Molecular Biology Reviews **72:**211-227.

18. **Jacobs C, Joris B, Jamin M, Klarsov K, Vanbeeumen J, Menginlecreulx D, Vanheijenoort J, Park JT, Normark S, Frere JM.** 1995. AmpD, essential for both beta-lactamase regulation and cell-wall recycling, is a novel cytosolic N-acetylmuramyl-L-alanine amidase. Molecular Microbiology **15:**553-559.
19. **Talfan A, Mounsey O, Charman M, Townsend E, Avison MB.** 2013. Involvement of mutation in *ampD* I, *mrcA*, and at least one additional gene in beta-lactamase hyperproduction in *Stenotrophomonas maltophilia*. Antimicrobial Agents and Chemotherapy **57:**5486-5491.
20. **Yang TC, Huang YW, Hu RM, Huang SC, Lin YT.** 2009. AmpD(I) is involved in expression of the chromosomal L1 and L2 beta-lactamases of *Stenotrophomonas maltophilia*. Antimicrobial Agents and Chemotherapy **53:**2902-2907.
21. **Lin CW, Lin HC, Huang YW, Chung TC, Yang TC.** 2011. Inactivation of *mrcA* gene derepresses the basal-level expression of L1 and L2 beta-lactamases in *Stenotrophomonas maltophilia*. Journal of Antimicrobial Chemotherapy **66:**2033-2037.
22. **Huang YW, Wu CJ, Hu RM, Lin YT, Yang TC.** 2015. Interplay among membrane-bound lytic transglycosylase D1, the CreBC two-component regulatory system, the AmpNG-AmpD(I)-NagZ-AmpR regulatory circuit, and L1/L2 beta-lactamase expression in *Stenotrophomonas maltophilia*. Antimicrobial Agents and Chemotherapy **59:**6866-6872.
23. **Bolger AM, Lohse M, Usadel B.** 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics **30:**2114-2120.
24. **Crossman LC, Gould VC, Dow JM, Vernikos GS, Okazaki A, Sebaihia M, Saunders D, Arrowsmith C, Carver T, Peters N, Adlem E, Kerhornou A, Lord A, Murphy L, Seeger K, Squares R, Rutter S, Quail MA, Rajandream MA, Harris D, Churcher C, Bentley SD, Parkhill J, Thomson NR, Avison MB.** 2008. The complete genome, comparative and functional analysis of *Stenotrophomonas maltophilia* reveals an organism heavily shielded by drug resistance determinants. Genome Biology **9**:R74.
25. **Darling AE, Mau B, Perna NT.** 2010. progressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. PLoS One **5:**e11147.
26. **Jain A, Srivastava P.** 2013. Broad host range plasmids. FEMS Microbiology Letters **348:**87-96.
27. **Obranic S, Babic F, Maravic-Vlahovfcek G.** 2013. Improvement of pBBR1MCS plasmids, a very useful series of broad-host-range cloning vectors. Plasmid **70:**263-267.
28. **Felici A, Amicosante G.** 1995. Kinetic analysis of extension of substrate specificity with *Xanthomonas maltophilia*, *Aeromonas hydrophila*, and *Bacillus cereus* metallo-beta-lactamases. Antimicrobial Agents and Chemotherapy **39:**192-199.
29. **Das D, Herve M, Feuerhelm J, Farr CL, Chiu HJ, Elsliger MA, Knuth MW, Klock HE, Miller MD, Godzik A, Lesley SA, Deacon AM, Mengin-Lecreulx D, Wilson IA.** 2011. Structure and function of the first full-length murein peptide ligase (Mpl) cell wall recycling protein. PLoS One **6:**e17624.
30. **Diaz Caballero J, Clark ST, Coburn B, Zhang Y, Wang PW, Donaldson SL, Tullis DE, Yau YCW, Waters VJ, Hwang DM, Guttman DS.** 2015. Selective sweeps and parallel pathoadaptation drive *Pseudomonas aeruginosa* evolution in the cystic fibrosis lung. MBio **6:** e00981-15.
31. **Williams D, Evans B, Haldenby S, Walshaw MJ, Brockhurst MA, Winstanley C, Paterson S.** 2015. Divergent, coexisting *Pseudomonas aeruginosa* lineages in chronic cystic fibrosis lung infections. American Journal of Respiratory and Critical Care Medicine **191:**775-785.
32. **Wang K, Chen YQ, Salido MM, Kohli GS, Kong JL, Liang HJ, Yao ZT, Xie YT, Wu HY, Cai SQ, Drautz-Moses DI, Darling AE, Schuster SC, Yang L, Ding YC.** 2017. The rapid in vivo evolution of *Pseudomonas aeruginosa* in ventilator-associated pneumonia patients leads to attenuated virulence. Open Biology **7**:pii:170029.
33. **Tsutsumi Y, Tomita H, Tanimoto K.** 2013. Identification of novel genes responsible for overexpression of *ampC* in *Pseudomonas aeruginosa* PAO1. Antimicrobial Agents and Chemotherapy **57:**5987-5993.
34. **Castanheira M, Mills JC, Farrell DJ, Jones RN.** 2014. Mutation-driven β-lactam resistance mechanisms among contemporary ceftazidime-nonsusceptible *Pseudomonas aeruginosa* isolates from U.S. hospitals. Antimicrob Agents Chemother. **58**:6844-50.

35. **Takebayashi Y, Wan Nur Ismah WAK, Findlay J, Heesom KJ, Zhang J, Williams M, MacGowan AP, Avison MB.** 2017. Prediction of cephalosporin and carbapenem susceptibility in multi-drug resistant Gram-negative bacteria using liquid chromatography-tandem mass spectrometry. **bioRxiv**:doi:10.1101/138594.